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OF  
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FOR  
MAMMALIAN SECRETORY PEPTIDE - 9

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## MAMMALIAN SECRETORY PEPTIDE - 9

The present application is a continuation of U.S. Patent Application Serial No. 09/318,028 filed May 25 1999, which is a continuation-in-part of U.S. Patent Application Serial No. 09/109,808 filed July 2, 1998, now abandoned, which claims the benefit of U.S. Provisional Application No. 60/089,899, filed June 17, 1998, U.S. Provisional Application No. 60/085,983 filed May 19, 1998 and U.S. Provisional Application No. 60/051,704 filed July 3, 1997.

## BACKGROUND OF THE INVENTION

Proliferation and differentiation of cells of multicellular organisms are controlled by hormones and polypeptide growth factors. These diffusable molecules allow cells to communicate with each other and act in concert to form cells and organs, and to repair and regenerate damaged tissue. Examples of hormones and growth factors include the steroid hormones (e.g. estrogen, testosterone), parathyroid hormone, follicle stimulating hormone, the interleukins, platelet derived growth factor (PDGF), epidermal growth factor (EGF), granulocyte-macrophage colony stimulating factor (GM-CSF), erythropoietin (EPO) and calcitonin.

Hormones and growth factors influence cellular metabolism by binding to proteins. Proteins may be integral membrane proteins that are linked to signaling pathways within the cell, such as second messenger

systems. Other classes of proteins are soluble molecules, such as the transcription factors.

Thus, there is a continuing need to discover new  
5 hormones, growth factors and the like.

#### SUMMARY OF THE INVENTION

The present invention addresses this need by  
10 providing a novel polypeptide and related compositions and methods. Within one aspect, the present invention provides an isolated polynucleotide encoding a mammalian polypeptide termed secretory peptide - 9, hereinafter referred to as Zsig9. The mature human Zsig9 polypeptide  
15 is comprised of a sequence of amino acids approximately 64 amino acids long. Amino acid residue 21 of SEQ ID NO: 2, an arginine, is the initial amino acid of the mature polypeptide. Thus, it is believed that amino residues 1-20 comprise a signal sequence, and the mature Zsig9  
20 polypeptide is represented by the amino acid sequence comprised of residues 21-84. The mature Zsig9 polypeptide is further represented by SEQ ID NO: 3. Within an additional embodiment, the polypeptide further comprises an affinity tag. Within a further embodiment, the  
25 polynucleotide is DNA.

Alternative forms of Zsig9 are defined by SEQ ID NOs: 4, 5, and 6. SEQ ID NO: 4 defines a processed form of Zsig9 in which the protein contains amino acid residues 23  
30 - 84 of SEQ ID NO: 2. SEQ ID NO: 5 represents another form of Zsig9 containing amino acid residues 23, a serine, to and including amino acid 47, a proline of SEQ ID NO: 2. SEQ ID NO: 6 defines another processed form of Zsig9 contain amino acid residues 50, a threonine, to and  
35 including amino acid 84 of SEQ ID NO: 2. SEQ ID NO: 16 and

17 represent another variant of Zsig9 and SEQ ID NO: 20 represents the mature sequence absent the first 20 amino acid residues, the signal sequence, of SEQ ID NO: 17.

SEQ ID NO: 18 and 19 represent the mouse ortholog of Zsig9; and SEQ ID NO: 21 depicts the mature amino acid sequence absent the first 20 amino acid residues, the signal sequence, of SEQ ID NO: 19.

Within a second aspect of the invention there is provided an expression vector comprising (a) a transcription promoter; (b) a DNA segment encoding a Zsig9 polypeptide as defined by SEQ ID NOs: 2-6, 17, 19, 20 and 21 or a polypeptide 90% identical to said polypeptides, and (c) a transcription terminator, wherein the promoter, DNA segment, and terminator are operably linked.

Within a third aspect of the invention there is provided a cultured eukaryotic cell into which has been introduced an expression vector as disclosed above, wherein said cell expresses a protein polypeptide encoded by the DNA segment.

Within a further aspect of the invention there is provided a chimeric polypeptide consisting essentially of a first portion and a second portion joined by a peptide bond. The first portion of the chimeric polypeptide consists essentially of (a) a Zsig9 polypeptide as shown in SEQ ID NOs: 2-6, 17, 19, 20 and 21(b) allelic variants of SEQ ID NOs: 2-6, 17, 19, 20 and 21; and (c) protein polypeptides that are at least 90% identical to (a) or (b). The second portion of the chimeric polypeptide consists essentially of another polypeptide such as an affinity tag. Within one

embodiment the affinity tag is an immunoglobulin F<sub>C</sub> polypeptide. The invention also provides expression vectors encoding the chimeric polypeptides and host cells transfected to produce the chimeric polypeptides.

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Within an additional aspect of the invention there is provided an antibody that specifically binds to a Zsig9 polypeptide as disclosed above, and also an anti-idiotypic antibody which neutralizes the antibody to a Zsig9 polypeptide.

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An additional embodiment of the present invention relates to a peptide or polypeptide which has the amino acid sequence of an epitope-bearing portion of a Zsig9 polypeptide having an amino acid sequence described above. Peptides or polypeptides having the amino acid sequence of an epitope-bearing portion of a Zsig9 polypeptide of the present invention include portions of such polypeptides with at least nine, preferably at least 15 and more preferably at least 30 to 50 amino acids, although epitope-bearing polypeptides of any length up to and including the entire amino acid sequence of a polypeptide of the present invention described above are also included in the present invention. An example of such a polypeptide is represented by SEQ ID NO:24.

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Also claimed are antibodies which bind specifically to the above-defined Zsig9 polypeptides, methods for making said antibodies, and anti-idiotypic antibodies of said Zsig9-binding antibodies.

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These and other aspects of the invention will become evident upon reference to the following detailed description.

## DETAILED DESCRIPTION OF THE INVENTION

5           The teachings of all of the references cited herein are incorporated in their entirety herein by reference.

10           The term "ortholog" (or "species homolog") denotes a polypeptide or protein obtained from one species that has homology to an analogous polypeptide or protein from a different species.

15           The term "paralog" denotes a polypeptide or protein obtained from a given species that has homology to a distinct polypeptide or protein from that same species.

20           The term "allelic variant" denotes any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequence.

25           The term allelic variant is also used herein to denote a protein encoded by an allelic variant of a gene.

30           The term "expression vector" denotes a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments include promoter and terminator sequences, and may optionally include one or more origins of replication, one or more selectable markers, an

35           enhancer, a polyadenylation signal and the like.

Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both.

The term "isolated", when applied to a  
5 polynucleotide, denotes that the polynucleotide has been removed from its natural genetic milieu and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are  
10 those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as  
15 promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art, Dynan and Tijan, *Nature* 316:774-78 (1985). When applied to a protein, the term "isolated" indicates that the protein is found in a condition other  
20 than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated protein is substantially free of other proteins, particularly other proteins of animal origin. It is preferred to provide the protein in a highly purified form, i.e.,  
25 greater than 95% pure, more preferably greater than 99% pure.

The term "operably linked", when referring to DNA segments, denotes that the segments are arranged so  
30 that they function in concert for their intended purposes, e.g. transcription initiates in the promoter and proceeds through the coding segment to the terminator.

The term "polynucleotide" is a single- or  
35 double-stranded polymer of deoxyribonucleotide or

ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules.

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The term "complements of polynucleotide molecules" denotes polynucleotide molecules having a complementary base sequence and reverse orientation as compared to a reference sequence.

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The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons (as compared to a reference polynucleotide molecule that encodes a polypeptide).

15 Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (i.e., GAU and GAC triplets each encode Asp).

20 The term "promoter" denotes a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

25 The term "secretory signal sequence" denotes a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger peptide  
30 is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

The term "receptor" denotes a cell-associated protein that binds to a bioactive molecule (i.e., a  
35 ligand) and mediates the effect of the ligand on the cell.



Membrane-bound receptors are characterized by a multi-domain structure comprising an extracellular ligand-binding domain and an intracellular effector domain that is typically involved in signal transduction. Binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell. This interaction in turn leads to an alteration in the metabolism of the cell. Metabolic events that are linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids. Most nuclear receptors also exhibit a multi-domain structure, including an amino-terminal, transactivating domain, a DNA binding domain and a ligand binding domain. In general, receptors can be membrane bound, cytosolic or nuclear; monomeric (e.g., thyroid stimulating hormone receptor, beta-adrenergic receptor) or multimeric (e.g., PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor).

The term "complement/anti-complement pair" denotes non-identical moieties that form a non-covalently associated, stable pair under appropriate conditions. For instance, biotin and avidin (or streptavidin) are prototypical members of a complement/anti-complement pair. Other exemplary complement/anti-complement pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs, sense/antisense polynucleotide pairs, and the like. Where subsequent dissociation of the complement/anti-complement pair is desirable, the

complement/anti-complement pair preferably has a binding affinity of  $<10^9 \text{ M}^{-1}$ .

A "soluble protein" is a protein polypeptide  
5 that is not bound to a cell membrane.

Within preferred embodiments of the invention the isolated polynucleotides will hybridize to similar sized regions of polynucleotide defined by SEQ ID NO:1, or  
10 a sequence complementary thereto, under highly stringent conditions. In general, stringent conditions are selected to be about  $5^\circ\text{C}$  lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength and pH. The  $T_m$  is the temperature (under defined ionic  
15 strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typical stringent conditions are those in which the salt concentration is about 0.02 M or less at pH 7 and the temperature is at least about  $60^\circ\text{C}$ . As previously noted,  
20 the isolated polynucleotides of the present invention include DNA and RNA. Methods for isolating DNA and RNA are well known in the art. Total RNA can be prepared using guanidine HCl extraction followed by isolation by centrifugation in a CsCl gradient, Chirgwin et al.,  
25 *Biochemistry* 18:52-94 (1979). Poly (A)<sup>+</sup> RNA is prepared from total RNA using the method of Aviv and Leder, *Proc. Natl. Acad. Sci. USA* 69:1408-1412 (1972). Complementary DNA (cDNA) is prepared from poly(A)<sup>+</sup> RNA using known methods. Polynucleotides encoding Zsig9 polypeptides are  
30 then identified and isolated by, for example, hybridization or PCR.

The present invention further provides counterpart polypeptides and polynucleotides from other species (orthologs or paralogs). Of particular interest are Zsig9 polypeptides from other mammalian species, including murine, rat, porcine, ovine, bovine, canine, feline, equine and other primate proteins. Species homologs of the human proteins can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses the protein. Suitable sources of mRNA can be identified by probing Northern blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line. A Zsig9-encoding cDNA can then be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequences. A cDNA can also be cloned using the polymerase chain reaction, or PCR (Mullis, U.S. Patent 4,683,202), using primers designed from the sequences disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to Zsig9. Similar techniques can also be applied to the isolation of genomic clones.

Those skilled in the art will recognize that the sequences disclosed in SEQ ID NOs:1 and 2, SEQ ID NOs:16 and 17, SEQ ID NOs. 18 and 19, represent a specific alleles of the human Zsig9 gene and polypeptide, and that allelic variation and alternative splicing are expected to occur. Allelic variants can be cloned by probing cDNA or genomic libraries from different individuals according to

standard procedures. Allelic variants of the DNA sequence shown in SEQ ID NO: 1, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NOS: 3, 4, 5, and 6.

Additionally, the polynucleotides of the present invention can be synthesized using DNA synthesizers. Currently the method of choice is the phosphoramidite method. Each complementary strand of a double stranded DNA of gene or a gene fragment is made separately. The production of short DNA fragments (60 to 80 bp) can be accomplished by synthesizing the complementary strands and then annealing them. For the production of longer DNA molecules the coupling efficiency of each cycle during chemical DNA synthesis is seldom 100%. To overcome this problem, synthetic genes (double-stranded) are assembled in modular form from single-stranded fragments that are from 20 to 100 nucleotides in length.

One method for building a synthetic gene requires the initial production of a set of overlapping, complementary oligonucleotides, each of which is between 20 to 60 nucleotides long. The sequences of the strands are planned so that, after annealing, the two end segments of the gene are aligned to give blunt ends. Each internal section of the gene has complementary 3' and 5' terminal extensions that are designed to base pair precisely with an adjacent section. Thus, after the gene is assembled, the only remaining requirement to complete the process is sealing the nicks along the backbones of the two strands with T4 DNA ligase. In addition to the protein coding sequence, synthetic genes can be designed with terminal sequences that facilitate insertion into a restriction

*Molecular Biotechnology, Principles & Applications of Recombinant DNA*, (ASM Press, Washington, D.C. 1994), Itakura, K. et al. Synthesis and use of synthetic oligonucleotides. *Annu. Rev. Biochem.* 53 : 323-356 (1984), and Climie, S. et al. Chemical synthesis of the thymidylate synthase gene. *Proc. Natl. Acad. Sci. USA* 87 :633-637 (1990).

Those skilled in the art will recognize that the sequences disclosed in SEQ ID NOS:1, 2, 3, 4, 5, and 6 represent single alleles of the human. Allelic variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals according to standard procedures.

The present invention further provides counterpart proteins and polynucleotides from other species ("species orthologs"). Of particular interest are Zsig9 polypeptides from other mammalian species, including murine, porcine, ovine, bovine, canine, feline, equine, and other primates. Species orthologs of the human Zsig9 protein can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses the protein. Suitable sources of mRNA can be identified by probing Northern blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line. A protein-encoding cDNA can then be isolated by a

variety of methods, such as by probing with a complete or partial human or mouse cDNA or with one or more sets of degenerate probes based on the disclosed sequences. A cDNA can also be cloned using the polymerase chain reaction, or PCR (Mullis, U.S. Patent No. 4,683,202), using primers designed from the sequences disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to the protein. Similar techniques can also be applied to the isolation of genomic clones. As used and claimed the language "an isolated polynucleotide which encodes a polypeptide, said polypeptide being defined by SEQ ID NO: 2-6, 17, 19, 20 and 21" includes all allelic variants and species orthologs of the polypeptide of SEQ ID NOs: 2-6, 17, 19, 20 and 21.

The present invention also provides isolated protein polypeptides that are substantially homologous to the polypeptides of SEQ ID NOs: 2, 3, 4, 5 or 6 and their species orthologs. By "isolated" is meant a protein or polypeptide that is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin. It is preferred to provide the polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. The term "substantially homologous" is used herein to denote polypeptides having 50%, preferably 60%, more preferably at least 80%, sequence identity to the sequence shown in SEQ ID NO:2, or its species orthologs. Such polypeptides will more preferably be at least 90% identical, and most preferably 95% or more identical to SEQ ID NO:3, or its species orthologs. Percent sequence

identity is determined by conventional methods. See, for example, Altschul et al., *Bull. Math. Bio.* 48: 603-616 (1986) and Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915-10919 (1992). Briefly, two amino acid

- 5 sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "BLOSUM 62" scoring matrix of Henikoff and Henikoff (ibid.) as shown in Table 2 (amino acids are indicated by the standard one-letter codes). The percent  
10 identity is then calculated as:

Total number of identical matches

x 100

[length of the longer sequence plus the  
number of gaps introduced into the longer  
15 sequence in order to align the two  
sequences]

Table 1

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-1	-2	-4	7						
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4



Those skilled in the art appreciate that there are many established algorithms to align two amino acid sequences. The "FASTA" similarity search algorithm of Pearson and Lipman is a suitable protein alignment method for examining the level of identity shared by an amino acid sequence and the amino acid sequence of a putative variant. The FASTA algorithm is described by Pearson and Lipman, *Proc. Nat'l Acad. Sci. USA* 85:2444 (1988), and by Pearson, *Meth. Enzymol.* 183:63 (1990). Briefly, FASTA first characterizes sequence similarity by identifying regions shared by the query sequence (e.g., SEQ ID NO:2) and a test sequence that have either the highest density of identities (if the ktup variable is 1) or pairs of identities (if ktup=2), without considering conservative amino acid substitutions, insertions or deletions. The ten regions with the highest density of identities are then rescored by comparing the similarity of all paired amino acids using an amino acid substitution matrix, and the ends of the regions are "trimmed" to include only those residues that contribute to the highest score. If there are several regions with scores greater than the "cutoff" value (calculated by a predetermined formula based upon the length of the sequence and the ktup value), then the trimmed initial regions are examined to determine whether the regions can be joined to form an approximate alignment with gaps. Finally, the highest scoring regions of the two amino acid sequences are aligned using a modification of the Needleman-Wunsch-Sellers algorithm (Needleman and Wunsch, *J. Mol. Biol.* 48:444 (1970); Sellers, *SIAM J. Appl. Math.* 26:787 (1974), which allows for amino acid insertions and deletions. Illustrative parameters for FASTA analysis are: ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=BLOSUM62. These parameters can be introduced into a FASTA program by

modifying the scoring matrix file ("SMATRIX"), as explained in Appendix 2 of Pearson, *Meth. Enzymol.* 183:63 (1990).

FASTA can also be used to determine the sequence identity of nucleic acid molecules using a ratio as disclosed above. For nucleotide sequence comparisons, the ktup value can range between one to six, preferably from four to six.

10           The present invention includes nucleic acid molecules that encode a polypeptide having one or more conservative amino acid changes, compared with the amino acid sequence of SEQ ID NO:3. The BLOSUM62 table is an amino acid substitution matrix derived from about 2,000  
15 local multiple alignments of protein sequence segments, representing highly conserved regions of more than 500 groups of related proteins [Henikoff and Henikoff, *Proc. Nat'l Acad. Sci. USA* 89:10915 (1992)]. Accordingly, the BLOSUM62 substitution frequencies can be used to define  
20 conservative amino acid substitutions that may be introduced into the amino acid sequences of the present invention. As used herein, the language "conservative amino acid substitution" refers to a substitution represented by a BLOSUM62 value of greater than -1. For  
25 example, an amino acid substitution is conservative if the substitution is characterized by a BLOSUM62 value of 0,1,2, or 3. Preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 1 (e.g., 1,2 or 3), while more preferred  
30 conservative substitutions are characterized by a BLOSUM62 value of at least 2 (e.g., 2 or 3). Accordingly the present invention claims those polypeptides which are at least 90%, preferably 95% and most preferably 99% identical to SEQ ID NO:3 and which are able to stimulate

antibody production in a mammal, and said antibodies are able to bind the native sequence of SEQ ID NO:3.

Substantially homologous proteins and

5 polypeptides are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions (see Table 2) and other

10 substitutions that do not significantly affect the folding or activity of the protein or polypeptide; small deletions, typically of one to about 30 amino acids; and small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that

15 facilitates purification (an affinity tag), such as a poly-histidine tract, protein A, Nilsson et al., *EMBO J.* 4:1075, (1985); Nilsson et al., *Methods Enzymol.* 198:3, (1991), glutathione S transferase, Smith and Johnson, *Gene* 67:31, (1988), or other antigenic epitope or binding

20 domain. See, in general Ford et al., *Protein Expression and Purification* 2: 95-107, (1991). DNAs encoding affinity tags are available from commercial suppliers (e.g., Pharmacia Biotech, Piscataway, NJ).

25 Table 2

Conservative amino acid substitutions

	Basic:	arginine
		lysine
		histidine
30	Acidic:	glutamic acid
		aspartic acid
	Polar:	glutamine
		asparagine
	Hydrophobic:	leucine

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Proteins are purified by chromatography. See, for example, Robertson *et al.*, *J. Am. Chem. Soc.* 113:2722 (1991); Ellman *et al.*, *Meth. Enzymol.* 202:301 (1991); Chung *et al.*, *Science* 259:806-09 (1993); and Chung *et al.*,  
 5 *Proc. Natl. Acad. Sci. USA* 90:10145-49 (1993). In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti *et al.*, *J. Biol. Chem.* 271:19991-98 (1996). Within a third method, *E. coli*  
 10 cells are cultured in the absence of a natural amino acid that is to be replaced (e.g., phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (e.g., 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, or 4-fluorophenylalanine). The non-  
 15 naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide *et al.*, *Biochem.* 33:7470-76, 1994. Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification.  
 20 Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, *Protein Sci.* 2:395-403 (1993).

A limited number of non-conservative amino  
 25 acids, amino acids that are not encoded by the genetic code, non-naturally occurring amino acids, and unnatural amino acids may be substituted for Zsig9\* amino acid residues. "Unnatural amino acids" have been modified after protein synthesis, and/or have a chemical structure  
 30 in their side chain(s) different from that of the standard amino acids. Unnatural amino acids can be chemically synthesized, or preferably, are commercially available, and include pipecolic acid, thiazolidine carboxylic acid,

dehydroproline, 3- and 4-methylproline, and 3,3-dimethylproline.

Essential amino acids in the polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis, Cunningham and Wells, *Science* 244, 1081-1085, (1989); Bass et al., *Proc. Natl. Acad. Sci. USA* 88:4498-4502, (1991). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (e.g., ligand binding and signal transduction) to identify amino acid residues that are critical to the activity of the molecule. Sites of ligand-protein interaction can also be determined by analysis of crystal structure as determined by such techniques as nuclear magnetic resonance, crystallography or photoaffinity labeling. See, for example, de Vos et al., *Science* 255:306-312, (1992); Smith et al., *J. Mol. Biol.* 224:899-904, (1992); Wlodaver et al., *FEBS Lett.* 309:59-64, (1992). The identities of essential amino acids can also be inferred from analysis of homologies with related proteins.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer, *Science* 241:53-57, (1988) or Bowie and Sauer, *Proc. Natl. Acad. Sci. USA* 86:2152-2156, (1989). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods

that can be used include phage display, e.g., Lowman et al., *Biochem.* 30:10832-10837, (1991); Ladner et al., U.S. Patent No. 5,223,409; Huse, WIPO Publication WO 92/06204, and region-directed mutagenesis, Derbyshire et al., *Gene* 5 46:145, (1986); Ner et al., *DNA* 7:127, (1988).

Mutagenesis methods as disclosed above can be combined with high-throughput screening methods to detect activity of cloned, mutagenized proteins in host cells.

10 Preferred assays in this regard include cell proliferation assays and biosensor-based ligand-binding assays, which are described below. Mutagenized DNA molecules that encode active proteins or portions thereof (e.g., ligand-binding fragments) can be recovered from the host cells and  
15 rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

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Using the methods discussed above, one of ordinary skill in the art can prepare a variety of polypeptides that are substantially homologous to SEQ ID NO:2 or allelic variants thereof and retain the properties  
25 of the wild-type protein. As expressed and claimed herein the language, "a polypeptide as defined by SEQ ID NO: 2" includes all allelic variants and species orthologs of the polypeptide.

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The protein/polypeptides of the present invention, including full-length proteins, protein fragments (e.g. ligand-binding fragments), and fusion polypeptides can be produced in genetically engineered host cells according to conventional techniques. Suitable

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or synthesized *de novo*. The secretory signal sequence is joined to the Zsig9 DNA sequence in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide of interest, although certain signal sequences may be positioned elsewhere in the DNA sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830).

Cultured mammalian cells are preferred hosts within the present invention. Methods for introducing exogenous DNA into mammalian host cells include calcium phosphate-mediated transfection, Wigler et al., *Cell* 14:725, (1978); Corsaro and Pearson, *Somatic Cell Genetics* 7:603, (1981); Graham and Van der Eb, *Virology* 52:456, (1973), electroporation, Neumann et al., *EMBO J.* 1:841-845, (1982), DEAE-dextran mediated transfection, Ausubel et al., eds., *Current Protocols in Molecular Biology*, John Wiley and Sons, Inc., NY, (1987), and liposome-mediated transfection, Hawley-Nelson et al., *Focus* 15:73, (1993); Ciccarone et al., *Focus* 15:80, (1993). The production of recombinant polypeptides in cultured mammalian cells is disclosed, for example, by Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821; and Ringold, U.S. Patent No. 4,656,134. Suitable cultured mammalian cells include the COS-1 (ATCC No. CRL 1650), COS-7 (ATCC No. CRL 1651), BHK (ATCC No. CRL 1632), BHK 570 (ATCC No. CRL 10314), 293, ATCC No. CRL 1573; Graham et al., *J. Gen. Virol.* 36:59-72, (1977) and Chinese hamster ovary (e.g. CHO-K1; ATCC No. CCL 61) cell lines. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, Rockville, Maryland. In general, strong transcription

metallothionein genes (U.S. Patent Nos. 4,579,821 and

Drug selection is generally used to select for cultured mammalian cells into which foreign DNA has been inserted. Such cells are commonly referred to as

"stable transfectants." A preferred selectable marker is a gene encoding resistance to the antibiotic neomycin.

type drug, such as G-418 or the like. Selection systems may also be used to increase the expression level of the gene of interest, a process referred to as

the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate.

Other higher eukaryotic cells can also be used as hosts, including insect cells, plant cells and avian cells. Transformation of insect cells and production of foreign polypeptides therein is disclosed by Guarino *et al.*, U.S. Patent No. 5,162,222; Bang *et al.*, U.S. Patent

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Fungal cells, including yeast cells, and particularly cells of the genus *Saccharomyces*, can also be used within the present invention, such as for producing protein fragments or polypeptide fusions. Methods for transforming yeast cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; Brake, U.S. Patent No. 4,870,008; Welch et al., U.S. Patent No. 5,037,743; and Murray et al., U.S. Patent No. 4,845,075. Transformed cells are selected by phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (e.g., leucine). A preferred vector system for use in yeast is the *POT1* vector system disclosed by Kawasaki et al. (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, e.g., Kawasaki, U.S. Patent No. 4,599,311; Kingsman et al., U.S. Patent No. 4,615,974; and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446; 5,063,154; 5,139,936 and 4,661,454. Transformation systems for other yeasts, including *Hansenula polymorpha*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Ustilago maydis*, *Pichia pastoris*, *Pichia methanolica*, *Pichia guilliermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson et al., *J. Gen. Microbiol.*

132:3459-3465, (1986) and Cregg, U.S. Patent No. 4,882,279.

Aspergillus cells may be utilized according to the methods of McKnight *et al.*, U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino *et al.*, U.S. Patent No. 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533.

Transformed or transfected host cells are cultured according to conventional procedures in a culture medium containing nutrients and other components required for the growth of the chosen host cells. A variety of suitable media, including defined media and complex media, are known in the art and generally include a carbon source, a nitrogen source, essential amino acids, vitamins and minerals. Media may also contain such components as growth factors or serum, as required. The growth medium will generally select for cells containing the exogenously added DNA by, for example, drug selection or deficiency in an essential nutrient which is complemented by the selectable marker carried on the expression vector or co-transfected into the host cell.

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Within one aspect of the present invention, a novel protein is produced by a cultured cell, and the cell is used to screen for a receptor or receptors for the protein, including the natural receptor, as well as

30 agonists and antagonists of the natural ligand.

Proteins of the present invention are useful for enhancing the growth or development of the placenta, heart, and liver. Zsig9 can be measured *in vitro* using

cultured cells or *in vivo* by administering molecules of the claimed invention to the appropriate animal model. For instance, Zsig9 transfected (or co-transfected) expression host cells may be embedded in an alginate environment and injected (implanted) into recipient animals. Alginate-poly-L-lysine microencapsulation, permselective membrane encapsulation and diffusion chambers have been described as a means to entrap transfected mammalian cells or primary mammalian cells.

10 These types of non-immunogenic "encapsulations" or microenvironments permit the transfer of nutrients into the microenvironment, and also permit the diffusion of proteins and other macromolecules secreted or released by the captured cells across the environmental barrier to the

15 recipient animal. Most importantly, the capsules or microenvironments mask and shield the foreign, embedded cells from the recipient animal's immune response. Such microenvironments can extend the life of the injected cells from a few hours or days (naked cells) to several

20 weeks (embedded cells).

Alginate threads provide a simple and quick means for generating embedded cells. The materials needed to generate the alginate threads are readily available and

25 relatively inexpensive. Once made, the alginate threads are relatively strong and durable, both *in vitro* and, based on data obtained using the threads, *in vivo*. The alginate threads are easily manipulable and the methodology is scaleable for preparation of numerous

30 threads. In an exemplary procedure, 3% alginate is prepared in sterile H<sub>2</sub>O, and sterile filtered. Just prior to preparation of alginate threads, the alginate solution is again filtered. An approximately 50% cell suspension (containing about  $5 \times 10^5$  to about  $5 \times 10^7$  cells/ml) is

mixed with the 3% alginate solution. One ml of the alginate/cell suspension is extruded into a 100 mM sterile filtered  $\text{CaCl}_2$  solution over a time period of ~15 min, forming a "thread". The extruded thread is then transferred into a solution of 50 mM  $\text{CaCl}_2$ , and then into a solution of 25 mM  $\text{CaCl}_2$ . The thread is then rinsed with deionized water before coating the thread by incubating in a 0.01% solution of poly-L-lysine. Finally, the thread is rinsed with Lactated Ringer's Solution and drawn from solution into a syringe barrel (without needle attached). A large bore needle is then attached to the syringe, and the thread is intraperitoneally injected into a recipient in a minimal volume of the Lactated Ringer's Solution.

An alternative *in vivo* approach for assaying proteins of the present invention involves viral delivery systems. Exemplary viruses for this purpose include adenovirus, herpesvirus, vaccinia virus and adeno-associated virus (AAV). Adenovirus, a double-stranded DNA virus, is currently the best studied gene transfer vector for delivery of heterologous nucleic acid (for a review, see T.C. Becker *et al.*, *Meth. Cell Biol.* 43:161-89 (1994); and J.T. Douglas and D.T. Curiel, *Science & Medicine* 4:44-53 (1997). The adenovirus system offers several advantages: adenovirus can (i) accommodate relatively large DNA inserts; (ii) be grown to high-titer; (iii) infect a broad range of mammalian cell types; and (iv) be used with a large number of available vectors containing different promoters. Also, because adenoviruses are stable in the bloodstream, they can be administered by intravenous injection. Some disadvantages (especially for gene therapy) associated with adenovirus gene delivery include: (i) very low efficiency integration into the host genome; (ii) existence in primarily episomal form; and

(iii) the host immune response to the administered virus, precluding readministration of the adenoviral vector.

By deleting portions of the adenovirus genome,  
5 larger inserts (up to 7 kb) of heterologous DNA can be  
accommodated. These inserts may be incorporated into the  
viral DNA by direct ligation or by homologous  
recombination with a co-transfected plasmid. In an  
exemplary system, the essential E1 gene has been deleted  
10 from the viral vector, and the virus will not replicate  
unless the E1 gene is provided by the host cell (i.e., the  
human 293 cell line). When intravenously administered to  
intact animals, adenovirus primarily targets the liver.  
If the adenoviral delivery system has an E1 gene deletion,  
15 the virus cannot replicate in the host cells. However,  
the host's tissue (i.e., liver) will express and process  
(and, if a signal sequence is present, secrete) the  
heterologous protein. Secreted proteins will enter the  
circulation in the highly vascularized liver, and effects  
20 on the infected animal can be determined.

The adenovirus system can also be used for  
protein production *in vitro*. By culturing adenovirus-  
infected non-293 cells under conditions where the cells  
25 are not rapidly dividing, the cells can produce proteins  
for extended periods of time. For instance, BHK cells are  
grown to confluence in cell factories, then exposed to the  
adenoviral vector encoding the secreted protein of  
interest. The cells are then grown under serum-free  
30 conditions, which allows infected cells to survive for  
several weeks without significant cell division.  
Alternatively, adenovirus vector infected 293S cells can  
be grown in suspension culture at relatively high cell  
density to produce significant amounts of protein (see A.

Garnier et al., *Cytotechnol.* 15:145-55 (1994). With either protocol, an expressed, secreted heterologous protein can be repeatedly isolated from the cell culture supernatant. Within the infected 293S cell production protocol, non-secreted proteins may also be effectively obtained.

#### PROTEIN ISOLATION:

Expressed recombinant polypeptides (or chimeric polypeptides) can be purified using fractionation and/or conventional purification methods and media. Ammonium sulfate precipitation and acid or chaotrope extraction may be used for fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable anion exchange media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and the like. PEI, DEAE, QAE and Q derivatives are preferred, with DEAE Fast-Flow Sepharose (Pharmacia, Piscataway, NJ) being particularly preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties. Examples of coupling



chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling

5 chemistries. These and other solid media are well known and widely used in the art, and are available from commercial suppliers. Methods for binding receptor polypeptides to support media are well known in the art. Selection of a particular method is a matter of routine

10 design and is determined in part by the properties of the chosen support. See, for example, *Affinity Chromatography: Principles & Methods*, Pharmacia LKB Biotechnology, Uppsala, Sweden, (1988).

15           The polypeptides of the present invention can be isolated by exploitation of their *properties*. For example, immobilized metal ion adsorption (IMAC) chromatography can be used to purify histidine-rich proteins. Briefly, a gel is first charged with divalent

20 metal ions to form a chelate, E. Sulkowski, *Trends in Biochem.* 3:1-7, (1985). Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong

25 chelating agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography, *Methods in Enzymol.*, Vol. 182, "Guide to Protein Purification", M. Deutscher, (ed.), Acad. Press, San Diego, (1990), pp.529-

30 39. Alternatively, a fusion of the polypeptide of interest and an affinity tag (e.g., polyhistidine, maltose-binding protein, an immunoglobulin domain) may be constructed to facilitate purification.

## Uses

Overexpression of Zsig9 in tumor cells indicates that Zsig9 can be used as an indicator for cancer. Thus, antibodies of Zsig9 can be used as a diagnostic to determine the presence of Zsig9 and thus the presence of cancer as explained below. Furthermore, antibodies labeled with radioisotopes or fused with toxins can be used as a therapeutic to treat cancer. Anti-sense nucleotides derived from the *Zsig9* cDNA can also be used as a therapeutic to inhibit the growth of tumor cells.

Suitable detectable molecules may be directly or indirectly attached to the polypeptide or antibody, and include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like. Suitable cytotoxic molecules may be directly or indirectly attached to the polypeptide or antibody, and include bacterial or plant toxins (for instance, diphtheria toxin, *Pseudomonas* exotoxin, ricin, abrin and the like), as well as therapeutic radionuclides, such as iodine-131, rhenium-188 or yttrium-90 (either directly attached to the polypeptide or antibody, or indirectly attached through means of a chelating moiety, for instance). Polypeptides or antibodies may also be conjugated to cytotoxic drugs, such as adriamycin. For indirect attachment of a detectable or cytotoxic molecule, the detectable or cytotoxic molecule may be conjugated with a member of a complementary/anticomplementary pair, where the other member is bound to the polypeptide or antibody portion. For these purposes, biotin/streptavidin is an exemplary complementary/anticomplementary pair.

Alternatively, if the polypeptide has multiple functional domains (i.e., an activation domain or a ligand binding domain, plus a targeting domain), a fusion protein including only the targeting domain may be suitable for directing a detectable molecule, a cytotoxic molecule or a complementary molecule to a cell or tissue type of interest. In instances where the domain only fusion protein includes a complementary molecule, the anti-complementary molecule may be conjugated to a detectable or cytotoxic molecule. Such domain-complementary molecule fusion proteins thus represent a generic targeting vehicle for cell/tissue-specific delivery of generic anti-complementary-detectable/ cytotoxic molecule conjugates.

In another embodiment, polypeptide-cytokine fusion proteins or antibody/fragment-cytokine fusion proteins may be used for enhancing *in vitro* cytotoxicity (for instance, that mediated by monoclonal antibodies against tumor targets) and for enhancing *in vivo* killing of target tissues (for example, blood and bone marrow cancers). See, generally, J.L. Hornick *et al.*, Blood 89:4437-47 (1997). In general, cytokines are toxic if administered systemically. The described fusion proteins enable targeting of a cytokine to a desired site of action, thereby providing an elevated local concentration of cytokine. Suitable Zsig9 polypeptides or anti-Zsig9 antibodies target an undesirable cell or tissue (i.e., a tumor or a leukemia), and the fused cytokine mediates improved target cell lysis by effector cells. Suitable cytokines for this purpose include interleukin 2 and

granulocyte-macrophage colony-stimulating factor (GM-CSF), for instance.

In yet another embodiment, if the Zsig9 polypeptide or anti-Zsig9 antibody targets vascular cells or tissues, such polypeptide or antibody may be conjugated with a radionuclide, and particularly with a beta-emitting radionuclide, to reduce restenosis. Such therapeutic approach poses less danger to clinicians who administer the radioactive therapy. For instance, iridium-192 impregnated ribbons placed into stented vessels of patients until the required radiation dose was delivered showed decreased tissue growth in the vessel and greater luminal diameter than the control group, which received placebo ribbons. Further, revascularisation and stent thrombosis were significantly lower in the treatment group. Similar results are predicted with targeting of a bioactive conjugate containing a radionuclide, as described herein.

The bioactive polypeptide or antibody conjugates described herein can be delivered intravenously, intraarterially or intraductally, or may be introduced locally at the intended site of action.

#### USES OF POLYNUCLEOTIDE/POLYPEPTIDE:

Molecules of the present invention can be used to identify and isolate receptors of Zsig9. For example, proteins and peptides of the present invention can be immobilized on a column and membrane preparations run over the column (*Immobilized Affinity Ligand Techniques*, Hermanson et al., eds., pp.195-202 (Academic Press, San Diego, CA, 1992)). Proteins and peptides can also be

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Transgenic mice, engineered to express the Zsig9 gene, and mice that exhibit a complete absence of Zsig9 gene function, referred to as "knockout mice", Snouwaert  
 5 et al., *Science* 257:1083 (1992), may also be generated, Lowell et al., *Nature* 366:740-42 (1993). These mice may be employed to study the Zsig9 gene and the protein encoded thereby in an *in vivo* system.

#### 10 CHROMOSOMAL LOCALIZATION:

Radiation hybrid mapping is a somatic cell genetic technique developed for constructing high-resolution, contiguous maps of mammalian chromosomes, Cox  
 15 et al., *Science* 250:245-50 (1990). Partial or full knowledge of a gene's sequence allows one to design PCR primers suitable for use with chromosomal radiation hybrid mapping panels. Commercially available radiation hybrid mapping panels which cover the entire human genome, such  
 20 as the Stanford G3 RH Panel and the GeneBridge 4 RH Panel (Research Genetics, Inc., Huntsville, AL), are available. These panels enable rapid, PCR-based chromosomal localizations and ordering of genes, sequence-tagged sites (STSs), and other nonpolymorphic and polymorphic markers  
 25 within a region of interest. This includes establishing directly proportional physical distances between newly discovered genes of interest and previously mapped markers. The precise knowledge of a gene's position can be useful for a number of purposes, including: 1)  
 30 determining if a sequence is part of an existing contig and obtaining additional surrounding genetic sequences in various forms, such as YACs, BACs or cDNA clones; 2) providing a possible candidate gene for an inheritable disease which shows linkage to the same chromosomal

region; and 3) cross-referencing model organisms, such as mouse, which may aid in determining what function a particular gene might have.

For pharmaceutical use, the proteins of the present invention are formulated for parenteral, particularly intravenous or subcutaneous, delivery according to conventional methods. Intravenous administration will be by bolus injection or infusion over a typical period of one to several hours. In general, pharmaceutical formulations will include a Zsig9 protein in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering agents, albumin to prevent protein loss on vial surfaces, etc. Methods of formulation are well known in the art and are disclosed, for example, in *Remington's Pharmaceutical Sciences*, 19<sup>th</sup> Edition Gennaro, ed., (Mack Publishing Co., Easton PA, 1995). Therapeutic doses will generally be in the range of 0.1 to 100  $\mu\text{g/kg}$  of patient weight per day, preferably 0.5-20  $\mu\text{g/kg}$  per day, with the exact dose determined by the clinician according to accepted standards, taking into account the nature and severity of the condition to be treated, patient traits, etc. Determination of dose is within the level of ordinary skill in the art. The proteins may be administered for acute treatment, over one week or less, often over a period of one to three days or may be used in chronic treatment, over several months or years. In general, a therapeutically effective amount of Zsig9 is an amount sufficient to produce a clinically significant change in the liver, kidney, heart or placenta.

### Zsig9 Structure

The potential N-terminal 3 dimensional structure of Zsig9 should have similarities to that predicted for amylin and calcitonin-gene related peptides (CGRP). Moving from the N-terminus to the C-terminus there is a predicted disulfide bond between residues 28 and 31 of SEQ ID NO:2. This is like the predicted disulfide of amylin (positions 35-40) and CGRP1 and 2 (positions 84-89). If the mature form of Zsig9 is not cleaved at the Lys-Lys at position 48-49, there is probably a beta turn followed by an extended region with hydrophobic residues packing along the hydrophobic face of the helix. The C-terminus is probably not amidated as in amylin and CGRP if the mature form is cleaved at the Lys-Lys at position 48-49. If the mature form ends at either the serine at position 81 or at the phenylalanine at position 83, then the glycine at position 82 or the glycine at position 84 may be used to amidate the C-terminus.

### Zsig9 Tissue Distribution/Multiple mRNA Sizes

Northern analysis shows that Zsig9 is ubiquitous with higher levels in placenta, pancreas, thyroid, prostate, and liver. This may indicate that it is involved in homeostasis. Zsig9 is transcribed by many cell types and is probably released by a variety of cell types in response to some metabolic stress. The Zsig9 receptor is then activated by Zsig9 to alleviate the stress. The stress may include non-optimal levels of sugar, carbohydrate, antigenic load, fat, temperature, pH, O<sub>2</sub>, osmotic concentration and the like.

Zsig9 is overexpressed in tumor cells. Thus, antibodies to Zsig9 can be used to diagnosis the presence



of tumors. Radiolabeled antibodies to Zsig9 can further be used as an imaging agent to locate and treat tumors in the body. Anti-sense nucleotides to *zsig9* can be used to inhibit the progression of tumors.

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## Therapeutic Utility

### Cancer Diagnosis and Therapy

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As can be seen in Example 5, Zsig9 is overexpressed in a number of human tumors including brain, liver, lung, esophageal, stomach, colon, rectal, thyroid, and lymphoma tumors. Thus, antibodies to Zsig9 can be used both to detect and treat the tumors which overexpress

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Zsig9. Radiolabeled antibodies to Zsig9 can be used both to detect and localize tumors. Antibodies which are either radiolabeled or to which a toxic polypeptide is fused can also be used to treat tumors which overexpress Zsig9.

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Nucleotide primers and probes of the *Zsig9* gene can be used to detect the overexpression of Zsig9 using PCR. Gene amplification of *Zsig9* can be determined indirectly by assay of a patient body fluid to detect the presence of elevated levels of Zsig9. Suitable body fluids include serum and urine and exudates of the putative tumor tissues. Examples of immunoassays which can be used in determining the expression of Zsig9 to diagnose neoplastic diseases are described in the patent and scientific literature. See, for example U.S. Patent Nos. 3,791,932;

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3,817,837; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; and 4,098,876. Antisense nucleotides to the *Zsig9* DNA and RNA can be administered to a patient

to inhibit expression of Zsig9 and thus inhibit tumor growth.

Antibodies to the Zsig9 polypeptide can be  
5 purified and then administered to a patient. These  
reagents can be combined for therapeutic use with  
additional active or inert ingredients, e.g., in  
pharmaceutically acceptable carriers or diluents along  
with physiologically innocuous stabilizers and excipients.  
10 These combinations can be sterile filtered and placed into  
dosage forms as by lyophilization in dosage vials or  
storage in stabilized aqueous preparations. This invention  
also contemplates use of antibodies, binding fragments  
thereof or single-chain antibodies of the antibodies  
15 including forms which are not complement binding.

The quantities of reagents necessary for  
effective therapy will depend upon many different factors,  
including means of administration, target site,  
20 physiological state of the patient, and other medications  
administered. Thus, treatment dosages should be titrated  
to optimize safety and efficacy. Typically, dosages used  
*in vitro* may provide useful guidance in the amounts useful  
for *in vivo* administration of these reagents. Animal  
25 testing of effective doses for treatment of particular  
disorders will provide further predictive indication of  
human dosage. Methods for administration include oral,  
intravenous, peritoneal, intramuscular, or transdermal  
administration. Pharmaceutically acceptable carriers will  
30 include water, saline, buffers to name just a few. Dosage  
ranges would ordinarily be expected from 1 $\mu$ g to 1000 $\mu$ g per  
kilogram of body weight per day. However, the doses may be  
higher or lower as can be determined by a medical doctor  
with ordinary skill in the art. For a complete discussion

of drug formulations and dosage ranges see *Remington's Pharmaceutical Sciences*, 19<sup>th</sup> Ed., (Mack Publishing Co., Easton, Penn., 1995), and *Goodman and Gilman's: The Pharmacological Bases of Therapeutics*, 9<sup>th</sup> Ed. (Pergamon Press 1996).

### Nucleic Acid-based Therapeutic Treatment

If a mammal has a mutated or lacks a Zsig9 gene, the Zsig9 gene can be introduced into the cells of the mammal. In one embodiment, a gene encoding a Zsig9 polypeptide is introduced *in vivo* in a viral vector. Such vectors include an attenuated or defective DNA virus, such as but not limited to herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral genes, are preferred. A defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector [Kaplitt et al., *Molec. Cell. Neurosci.*, 2 :320-330 (1991)], an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al., *J. Clin. Invest.*, 90 :626-630 (1992), and a defective adeno-associated virus vector [Samulski et al., *J. Virol.*, 61:3096-3101 (1987); Samulski et al. *J. Virol.*, 63:3822-3828 (1989)].

In another embodiment, the gene can be introduced in a retroviral vector, e.g., as described in Anderson et al., U.S. Patent No. 5,399,346; Mann et al.,

Cell, 33:153 (1983); Temin et al., U.S. Patent No. 4,650,764; Temin et al., U.S. Patent No. 4,980,289; Markowitz et al., *J. Virol.*, 62:1120 (1988); Temin et al., U.S. Patent No. 5,124,263; International Patent  
 5 Publication No. WO 95/07358, published March 16, 1995 by Dougherty et al.; and *Blood*, 82:845 (1993).

Alternatively, the vector can be introduced by lipofection *in vivo* using liposomes. Synthetic cationic  
 10 lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker [Felgner et al., *Proc. Natl. Acad. Sci. USA*, 84:7413-7417 (1987); see Mackey et al., *Proc. Natl. Acad. Sci. USA*, 85:8027-8031 (1988)]. The use of lipofection to introduce exogenous  
 15 genes into specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. It is clear that directing transfection to particular cells represents one area of benefit. It is clear that directing transfection  
 20 to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides, e.g., hormones or  
 25 neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

It is possible to remove the cells from the body  
 30 and introduce the vector as a naked DNA plasmid and then re-implant the transformed cells into the body. Naked DNA vector for gene therapy can be introduced into the desired host cells by methods known in the art, e.g., transfection, electroporation, microinjection,

transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun or use of a DNA vector transporter [see, e.g., Wu et al., *J. Biol. Chem.*, 267:963-967 (1992); Wu et al., *J. Biol. Chem.*, 263:14621-14624 (1988)].

Zsig9 polypeptides can also be used to prepare antibodies that specifically bind to Zsig9 epitopes, peptides or polypeptides. The Zsig9 polypeptide or a fragment thereof is inoculate into an animal so as to elicit an immune response. Antibodies generated from this immune response can be isolated and purified as described herein. Methods for preparing and isolating polyclonal and monoclonal antibodies are well known in the art. See, for example, *Current Protocols in Immunology*, Cooligan, et al., Eds., (National Institutes of Health, John Wiley and Sons, Inc., 1995); Sambrook et al., *Molecular Cloning: A Laboratory Manual, Second Edition*, (Cold Spring Harbor, NY, 1989); and Hurrell, J. G. R., Ed., *Monoclonal Hybridoma Antibodies: Techniques and Applications*, (CRC Press, Inc., Boca Raton, FL, 1982).

As would be evident to one of ordinary skill in the art, polyclonal antibodies can be generated from inoculating a variety of warm-blooded animals such as horses, cows, goats, sheep, dogs, chickens, rabbits, mice, and rats with a Zsig9 polypeptide or a fragment thereof. The immunogenicity of a Zsig9 polypeptide may be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of Zsig9 or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-

length molecule or a portion thereof. If the polypeptide portion is "hapten-like", such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

As used herein, the term "antibodies" includes polyclonal antibodies, affinity-purified polyclonal antibodies, monoclonal antibodies, and antigen-binding fragments, such as  $F(ab')_2$  and Fab proteolytic fragments. Genetically engineered intact antibodies or fragments, such as chimeric antibodies, Fv fragments, single chain antibodies and the like, as well as synthetic antigen-binding peptides and polypeptides, are also included.

Non-human antibodies may be humanized by grafting non-human CDRs onto human framework and constant regions, or by incorporating the entire non-human variable domains (optionally "cloaking" them with a human-like surface by replacement of exposed residues, wherein the result is a "veneered" antibody). In some instances, humanized antibodies may retain non-human residues within the human variable region framework domains to enhance proper binding characteristics. Through humanizing antibodies, biological half-life may be increased, and the potential for adverse immune reactions upon administration to humans is reduced.

Alternative techniques for generating or selecting antibodies useful herein include *in vitro* exposure of lymphocytes to Zsig9 protein or peptide, and selection of antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled Zsig9 protein or peptide). Genes encoding polypeptides having potential Zsig9 polypeptide binding

domains can be obtained by screening random peptide libraries displayed on phage (phage display) or on bacteria, such as *E. coli*. Nucleotide sequences encoding the polypeptides can be obtained in a number of ways, such as through random mutagenesis and random polynucleotide synthesis. These random peptide display libraries can be used to screen for peptides which interact with a known target which can be a protein or polypeptide, such as a ligand or receptor, a biological or synthetic macromolecule, or organic or inorganic substances.

Techniques for creating and screening such random peptide display libraries are known in the art (Ladner et al., US Patent NO. 5,223,409; Ladner et al., US Patent NO. 4,946,778; Ladner et al., US Patent NO. 5,403,484 and Ladner et al., US Patent NO. 5,571,698) and random peptide display libraries and kits for screening such libraries are available commercially, for instance from Clontech (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA) and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Random peptide display libraries can be screened using the Zsig9 sequences disclosed herein to identify proteins which bind to Zsig9. These "binding proteins" which interact with Zsig9 polypeptides can be used for tagging cells; for isolating homolog polypeptides by affinity purification; they can be directly or indirectly conjugated to drugs, toxins, radionuclides and the like. These binding proteins can also be used in analytical methods such as for screening expression libraries and neutralizing activity. The binding proteins can also be used for diagnostic assays for determining circulating levels of polypeptides; for detecting or quantitating soluble polypeptides as marker of underlying pathology or disease.

These binding proteins can also act as Zsig9 "antagonists" to block Zsig9 binding and signal transduction *in vitro* and *in vivo*. These anti-Zsig9 binding proteins would be useful for inhibiting the growth of tumors.

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Antibodies are determined to be specifically binding if: 1) they exhibit a threshold level of binding activity, and 2) they do not significantly cross-react with related polypeptide molecules. First, antibodies  
 10 herein specifically bind if they bind to a Zsig9 polypeptide, peptide or epitope with a binding affinity ( $K_a$ ) of  $10^6 \text{ M}^{-1}$  or greater, preferably  $10^7 \text{ M}^{-1}$  or greater, more preferably  $10^8 \text{ M}^{-1}$  or greater, and most preferably  $10^9 \text{ M}^{-1}$  or greater. The binding affinity of an antibody can  
 15 be readily determined by one of ordinary skill in the art, for example, by Scatchard analysis, Scatchard, G., *Ann. NY Acad. Sci.* 51: 660-672 (1949).

Second, antibodies are determined to  
 20 specifically bind if they do not significantly cross-react with related polypeptides. Antibodies do not significantly cross-react with related polypeptide molecules, for example, if they detect Zsig9 but not known related polypeptides using a standard Western blot analysis  
 25 (Ausubel *et al.*, *ibid.*). Examples of known related polypeptides are orthologs, proteins from the same species that are members of a protein family (e.g. IL-16), Zsig9 polypeptides, and non-human Zsig9. Moreover, antibodies may be "screened against" known related polypeptides to  
 30 isolate a population that specifically binds to the inventive polypeptides. For example, antibodies raised to Zsig9 are adsorbed to related polypeptides adhered to insoluble matrix; antibodies specific to Zsig9 will flow



through the matrix under the proper buffer conditions. Such screening allows isolation of polyclonal and monoclonal antibodies non-crossreactive to closely related polypeptides, *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), (Cold Spring Harbor Laboratory Press, 1988); *Current Protocols in Immunology*, Cooligan, et al. (eds.), (National Institutes of Health, John Wiley and Sons, Inc., 1995). Screening and isolation of specific antibodies is well known in the art. See, *Fundamental Immunology*, Paul Eds., (Raven Press, 1993); Getzoff et al., *Adv. in Immunol.* 43: 1-98 (1988); *Monoclonal Antibodies: Principles and Practice*, Goding, J.W., Eds., (Academic Press Ltd., 1996); Benjamin et al., *Ann. Rev. Immunol.* 2: 67-101 (1984).

A variety of assays known to those skilled in the art can be utilized to detect antibodies which specifically bind to Zsig9 proteins or peptides. Exemplary assays are described in detail in *Antibodies: A Laboratory Manual*, Harlow and Lane, Eds., (Cold Spring Harbor Laboratory Press, 1988). Representative examples of such assays include: concurrent immunoelectrophoresis, radioimmunoassay, radioimmuno-precipitation, enzyme-linked immunosorbent assay (ELISA), dot blot or Western blot assay, inhibition or competition assay, and sandwich assay. In addition, antibodies can be screened for binding to wild-type versus mutant Zsig9 protein or polypeptide.

Antibodies to Zsig9 may be used for tagging cells that express Zsig9; for isolating Zsig9 by affinity purification; for diagnostic assays for determining circulating levels of Zsig9 polypeptides; for detecting or quantitating soluble Zsig9 as marker of underlying

pathology or disease; in analytical methods employing FACS; for screening expression libraries; for generating anti-idiotypic antibodies; and as neutralizing antibodies or as antagonists to block Zsig9 *in vitro* and *in vivo*.

5 Suitable direct tags or labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like; indirect tags or labels may feature use of biotin-avidin or other complement/anti-complement pairs as  
 10 intermediates. Antibodies herein may also be directly or indirectly conjugated to drugs, toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications. Moreover, antibodies to Zsig9 or fragments thereof may be used *in vitro* to detect  
 15 denatured Zsig9 or fragments thereof in assays, for example, Western Blots or other assays known in the art.

Another embodiment of the present invention provides for a peptide or polypeptide comprising an  
 20 epitope-bearing portion of a polypeptide of the invention. The epitope of the this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. A region of a protein to which an antibody can bind is defined as an "antigenic epitope". See for  
 25 instance, Geysen, H.M. *et al.*, *Proc. Natl. Acad Sci. USA* 81:3998-4002 (1984).

As to the selection of peptides or polypeptides bearing an antigenic epitope (*i.e.*, that contain a region  
 30 of a protein molecule to which an antibody can bind), it is well known in the art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See Sutcliffe, J.G.

*et al. Science* 219:660-666 (1983). Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined

5 neither to immunodominant regions of intact proteins (*i.e.*, immunogenic epitopes) nor to the amino or carboxyl terminals. Peptides that are extremely hydrophobic and those of six or fewer residues generally are ineffective at inducing antibodies that bind to the mimicked protein;  
10 longer soluble peptides, especially those containing proline residues, usually are effective.

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to  
15 raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. Antigenic epitope-bearing peptides and polypeptides of the present invention contain a sequence of at least nine, preferably between 15 to about 30 amino acids contained  
20 within the amino acid sequence of a polypeptide of the invention. However, peptides or polypeptides comprising a larger portion of an amino acid sequence of the invention, containing from 30 to 50 amino acids, or any length up to and including the entire amino acid sequence of a  
25 polypeptide of the invention, also are useful for inducing antibodies that react with the protein. Preferably, the amino acid sequence of the epitope-bearing peptide is selected to provide substantial solubility in aqueous solvents (*i.e.*, the sequence includes relatively  
30 hydrophilic residues and hydrophobic residues are preferably avoided); and sequences containing proline residues are particularly preferred. All of the polypeptides shown in the sequence listing contain

antigenic epitopes to be used according to the present invention.

#### BIOACTIVE CONJUGATES:

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Antibodies or polypeptides herein can also be directly or indirectly conjugated to drugs, toxins, radionuclides and the like, and these conjugates used for *in vivo* diagnostic or therapeutic applications. For instance, polypeptides or antibodies of the present invention can be used to identify or treat tissues or organs that express a corresponding anti-complementary molecule (receptor or antigen, respectively, for instance). More specifically, Zsig9 polypeptides or anti-  
10 Zsig9 antibodies, or bioactive fragments or portions thereof, can be coupled to detectable or cytotoxic molecules and delivered to a mammal having cells, tissues or organs that express the anti-complementary molecule.

Suitable detectable molecules may be directly or indirectly attached to the polypeptide or antibody, and include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent markers, chemiluminescent markers, magnetic particles and the like. Suitable cytotoxic  
20 molecules may be directly or indirectly attached to the polypeptide or antibody, and include bacterial or plant toxins (for instance, diphtheria toxin, *Pseudomonas* exotoxin, ricin, abrin and the like), as well as therapeutic radionuclides, such as iodine-131, rhenium-188  
25 or yttrium-90 (either directly attached to the polypeptide or antibody, or indirectly attached through means of a chelating moiety, for instance). Polypeptides or antibodies may also be conjugated to cytotoxic drugs, such as adriamycin. For indirect attachment of a detectable or  
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cytotoxic molecule, the detectable or cytotoxic molecule can be conjugated with a member of a complementary/ anticomplementary pair, where the other member is bound to the polypeptide or antibody portion. For these purposes,  
 5 biotin/streptavidin is an exemplary complementary/ anticomplementary pair.

In another embodiment, polypeptide-toxin fusion proteins or antibody-toxin fusion proteins can be used for  
 10 targeted cell or tissue inhibition or ablation (for instance, to treat cancer cells or tissues). Alternatively, if the polypeptide has multiple functional domains (i.e., an activation domain or a ligand binding domain, plus a targeting domain), a fusion protein  
 15 including only the targeting domain may be suitable for directing a detectable molecule, a cytotoxic molecule or a complementary molecule to a cell or tissue type of interest. In instances where the domain only fusion protein includes a complementary molecule, the anti-  
 20 complementary molecule can be conjugated to a detectable or cytotoxic molecule. Such domain-complementary molecule fusion proteins thus represent a generic targeting vehicle for cell/tissue-specific delivery of generic anti-complementary-detectable/ cytotoxic molecule conjugates.

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In another embodiment, Zsig9-cytokine fusion proteins or antibody-cytokine fusion proteins can be used for enhancing *in vivo* killing of target tissues (for example, blood and bone marrow cancers), if the Zsig9  
 30 polypeptide or anti-Zsig9 antibody targets the hyperproliferative blood or bone marrow cell (See, generally, Hornick *et al.*, *Blood* 89:4437-47 (1997)). They described fusion proteins enable targeting of a cytokine to a desired site of action, thereby providing an elevated

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USES OF POLYNUCLEOTIDE/POLYPEPTIDE:

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1990) or photoaffinity labeled, Brunner *et al.*, *Ann. Rev. Biochem.* 62:483-514 1993 and Fedan *et al.*, *Biochem. Pharmacol.* 33:1167-80 (1984) and specific cell-surface proteins can be identified.

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#### GENE THERAPY:

Polynucleotides encoding Zsig9 polypeptides are useful within gene therapy applications where it is  
 10 desired to increase or inhibit Zsig9 activity. If a mammal has a mutated or absent Zsig9 gene, the Zsig9 gene can be introduced into the cells of the mammal. In one embodiment, a gene encoding a Zsig9 polypeptide is introduced *in vivo* in a viral vector. Such vectors  
 15 include an attenuated or defective DNA virus, such as, but not limited to, herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, which entirely or almost entirely lack viral  
 20 genes, are preferred. A defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Examples of particular vectors include, but  
 25 are not limited to, a defective herpes simplex virus 1 (HSV1) vector (Kaplitt *et al.*, *Molec. Cell. Neurosci.* 2:320-30 (1991); an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet *et al.*, *J. Clin. Invest.* 90:626-30 (1992); and a defective adeno-  
 30 associated virus vector, Samulski *et al.*, *J. Virol.* 61:3096-101 (1987); Samulski *et al.*, *J. Virol.* 63:3822-8 (1989).

In another embodiment, a Zsig9 gene can be introduced in a retroviral vector, *e.g.*, as described in

Anderson et al., U.S. Patent No. 5,399,346; Mann et al. Cell 33:153 (1983); Temin et al., U.S. Patent No. 4,650,764; Temin et al., U.S. Patent No. 4,980,289; Markowitz et al., *J. Virol.* 62:1120 (1988); Temin et al., U.S. Patent No. 5,124,263; International Patent Publication No. WO 95/07358, published March 16, 1995 by Dougherty et al.; and Kuo et al., *Blood* 82:845 (1993). Alternatively, the vector can be introduced by lipofection *in vivo* using liposomes. Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7 (1987); Mackey et al., *Proc. Natl. Acad. Sci. USA* 85:8027-31 (1988). The use of lipofection to introduce exogenous genes into specific organs *in vivo* has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. More particularly, directing transfection to particular cells represents one area of benefit. For instance, directing transfection to particular cell types would be particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides (e.g., hormones or neurotransmitters), proteins such as antibodies, or non-peptide molecules can be coupled to liposomes chemically.

It is possible to remove the target cells from the body; to introduce the vector as a naked DNA plasmid; and then to re-implant the transformed cells into the body. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, e.g., transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran,



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The invention is further illustrated by the following non-limiting examples.

#### Example 1

##### Cloning of Zsig9

5 Zsig9 was identified from expressed sequence tag (EST) SEQ ID NO: 7. The cDNA clone containing the EST was discovered in a placenta from a full-term pregnancy cDNA library which contained the EST. The cDNA was isolated from *E. coli* transfected with the plasmid and then streaked out on an LB 100 µg/ml ampicillin and 100 µg/ml methicillin plate. The cDNA insert was sequenced. The insert was determined to be 649 base pairs long with a 84 amino acid open reading frame and a putative 20 amino acid signal peptide.

#### Example 2

##### Construction of Zsig9 Expression Vectors

20 Two Zsig9 construction vectors were made in a flag amino acid sequence (SEQ ID NO: 8) was inserted onto the N-terminal or C-terminal ends of the Zsig9 polypeptide. For the construction in which the flag amino acid sequence was attached to the N-terminus of Zsig9, a 25 473 bp Zsig9 PCR DNA fragment was generated with 1 µl of a ¼ dilution of the plasmid prep of Example 1 and 1 microliter (µl) of SEQ ID NO: 9 and 10 each having a concentration of 20 picomoles (pm)/µl of primer. The PCR mixture contained 2.5 µl of 10X PCR buffer, 0.5 KLENTAQ (both from CLONTECH), 2.5 µl REDI-LOAD dye (Research Genetics), 2.5 nucleotide triphosphate mix (Perkin-Elmer) and 14 µl of water. The PCR reaction was incubated at 94°C

for 5 minutes, and then run for 10 cycles each individual cycle being comprised of 30 seconds at 94°C and 2 minutes at 75°C. This was followed by 15 cycles each cycle being comprised of 30 seconds at 94°C and 2 minutes at 60°C. The reaction was ended with an incubation for 10 minutes at 74°C.

The resultant PCR mixture was then run on a 0.9% LMP agarose gel with TBE buffer. After the gel was run the band containing the DNA was cut out and the DNA was purified from the gel with a QUIAQUICK® column (Qiagen). 100 µl of the DNA was digested in a solution containing 4 µl of B buffer, 1 µl of *Bam*H1 (Boehringer Mannheim) and 1 µl of *Xho*1 (Boehringer Mannheim) for 2 hours at 37°C. The digested reaction mixture was electrophoresed on a 1% TBE gel; the DNA band was excised with a razor blade and the DNA was extracted from the gel with the Qiaquick® Gel Extraction Kit (Qiagen).

The excised DNA was subcloned into plasmid NF/pZP9 which had been cut with *Bam* and *Xho*. NF/pZP9 is a mammalian cell expression vector comprising an expression cassette containing the mouse metallothionein-1 promoter, a sequence encoding the tissue plasminogen activator (TPA) leader, then the flag peptide (SEQ ID NO:8), then multiple restriction sites. These were followed by the human growth hormone terminator, an *E. coli* origin of replication and a mammalian selectable marker expression unit containing the SV40 promoter, enhancer and origin of replication; a dihydrofolate reductase gene (DHFR) and the SV40 terminator. 1 µl containing 10 ng of the NF/pZP9 vector which had been previously digested with *Xho* and *Bam*HI was mixed with 1 µl of 10X ligase buffer, 1 µl of T4 ligase and

2 µl of Zsig9 fragment containing 20 ng. The ligation took place at room temperature for 3 hours and then electroporated into DH10b cells. After the electroporation the cells were plated onto LB-amp plates.

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For the construction of the Zsig9 gene in which the flag polypeptide SEQ ID NO: 8 was inserted onto the C-terminus of the Zsig9 polypeptide, a 649 bp Zsig9 PCR fragment was generated with 1 µl of ¼ dilution of the plasmid preparation containing Zsig9 described in Example 1 and 20 pm each of primers SEQ ID NO: 11 and SEQ ID NO: 12. The PCR reaction was incubated at 94°C for 5 minutes, then run for 10 cycles, each cycle being comprised 30 seconds at 94°C and 2 minutes at 75°C. This was followed by 15 cycles each cycle comprised of 30 seconds at 94°C and 2 minutes at 60°C. The reaction was ended with a final 10 minute extension at 74°C.

The entire reaction mixture was run on a 1% TBE gel and the DNA was cut out with a razor blade and the DNA was extracted using the QIAQUICK™ gel extraction kit. 20 µl out of the recovered 35 µl digested with 10 units of *Bam*H1 (Boehringer Mannheim) and 10 units of *Eco*R1 (Gibco BRL) for 2 hours at 37°C. The digested PCR mixture was electrophoresed on a 1% TBE gel. The DNA band was cut out with a razor blade and the DNA was extracted from the gel using the QIAquick® Gel Extraction Kit (Qiagen). The extracted DNA was subcloned into plasmid CF/pZP9 which had been cut with *Eco*R1 and *Bam*H1. Plasmid cfpzp9 is a mammalian expression vector containing an expression cassette having the mouse metallothionein-1 promoter, multiple restriction sites for insertion of coding

sequences, a sequence encoding the flag peptide, SEQ ID NO:10, a stop codon, a human growth hormone terminator, an *E. coli* origin of replication, a mammalian selectable marker expression unit having an SV40 promoter, enhancer and origin of replication, a DHFR gene and the SV40 terminator.

### Example 3 Tissue Distribution

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Human Multiple Tissue Northern Blots (MTN I, MTN II, and MTN III; Clontech) were probed to determine the tissue distribution of human ZSIG-9 expression. A 40 bp probe (SEQ ID NO: 13) was used to probe the blots.

15 The 5' end of the probe was radioactively labeled using T4 polynucleotide kinase and forward reaction buffer (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's specifications. The probe was purified using a NUCTRAP push column (Stratagene, La Jolla, CA). ExpressHyb™

20 (Clontech) solution was used for prehybridization and as a hybridizing solution for the Northern blots.

Hybridization took place overnight at 42°C using  $2 \times 10^6$  cpm/ml of labeled probe. The blots were then washed at 55°C in 1X SSC, 0.1% SDS. A 1.2 kb transcript was

25 detected. The signal was strongest in heart, placenta, liver and kidney. An intermediate signal was detected in spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood lymphocytes, thyroid, spinal cord. Weak signal was detected in lymph node, trachea, adrenal gland and bone marrow.

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EXAMPLE 4Chromosomal Assignment and Placement of Zsig9

Zsig9 was mapped to chromosome 12 using the  
5 commercially available "GeneBridge 4 Radiation Hybrid  
Panel" (Research Genetics, Inc., Huntsville, AL). The  
GeneBridge 4 Radiation Hybrid Panel contains PCRable DNAs  
from each of 93 radiation hybrid clones, plus two control  
DNAs (the HFL donor and the A23 recipient). A publicly  
10 available WWW server ([http://www-genome.wi.mit.edu/cgi-](http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl)  
[bin/contig/rhmapper.pl](http://www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl)) allows mapping relative to the  
Whitehead Institute/MIT Center for Genome Research's  
radiation hybrid map of the human genome (the "WICGR"  
radiation hybrid map) which was constructed with the  
15 GeneBridge 4 Radiation Hybrid Panel.

For the mapping of Zsig9 with the "GeneBridge 4  
RH Panel", 20 µl reactions were set up in a PCRable 96-  
well microtiter plate (Stratagene, La Jolla, CA) and used  
20 in a "RoboCycler Gradient 96" thermal cycler (Stratagene).  
Each of the 95 PCR reactions consisted of 2 µl 10X KlenTaq  
PCR reaction buffer (CLONTECH Laboratories, Inc., Palo  
Alto, CA), 1.6 µl dNTPs mix (2.5 mM each, PERKIN-ELMER,  
Foster City, CA), 1 µl sense primer, SEQ ID NO: 14, 1 µl  
25 antisense primer, SEQ ID NO: 15, 2 µl "RediLoad"  
(Research Genetics, Inc., Huntsville, AL), 0.4 µl 50X  
Advantage KlenTaq Polymerase Mix (Clontech Laboratories,  
Inc.), 25 ng of DNA from an individual hybrid clone or  
control and x µl ddH<sub>2</sub>O for a total volume of 20 µl. The  
30 reactions were overlaid with an equal amount of mineral  
oil and sealed. The PCR cycler conditions were as follows:  
an initial 1 cycle 5 minute denaturation at 95°C, 35 cycles  
of a 1 minute denaturation at 95°C, 1 minute annealing at  
62°C and 1.5 minute extension at 72°C, followed by a final

1 cycle extension of 7 minutes at 72°C. The reactions were separated by electrophoresis on a 3% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME).

The results showed that Zsig9 maps 344.72 cR\_3000 from the top of the human chromosome 12 linkage group on the WICGR radiation hybrid map. Proximal and distal framework markers were WI-6672 (D12S1410) and RP\_L41\_1, respectively. The use of the surrounding markers positions Zsig9 in the 12q15 region on the integrated LDB chromosome 12 map (The Genetic Location Database, University of Southampton, WWW server: [http://cedar.genetics.soton.ac.uk/public\\_html/](http://cedar.genetics.soton.ac.uk/public_html/)).

### Example 5

## Northern Blot Analysis of Human Tumors

Northern Analysis was carried out on the following Tumor Blots ; Human Tumor Panel Blot I, Human Tumor Panel Blot II, Human Tumor Panel Blot V, Human Stomach Tumor Blot, and Human Colon Tumor Blot (Clontech, Palo Alto, California). A probe was obtained using a PCR product representing the full length coding sequence of zsig9.

The probe was radioactively labeled with  $^{32}\text{P}$  using Rediprime Labeling System from Amersham (England). The probe was purified using a NUCTRAP push column (Stratagene Cloning Systems, La Jolla, Ca.) . EXPRESSHYB (Clontech, Palo Alto, Ca.) solution was used for prehybridization and hybridization. The hybridization solution consisted of 8 mls EXPRESSHYB, 80  $\mu\text{l}$  Sheared Salmon Sperm DNA (10mg/ml, 5 Prime-3 Prime, Boulder, CO) , 48  $\mu\text{l}$  Human Cot-1 DNA (1mg/ml, Gibco BRL) and 20  $\mu\text{l}$  labeled probe ( $8 \times 10^{-5}$  CPM/ $\mu\text{l}$ ). Hybridization took place

overnight at 55°C And the blots were then washed in 2X SSC,0.1%SDS at RT, then 2X SSC,0.1% SDS at 60°C, followed by 0.1X SSC, 0.1% SDS wash at 60°C. The blots were exposed overnight and developed.

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A transcript of .8-1kb was observed in the following tissues. The strongest signals were in brain tumor, liver tumor, esophageal tumor, stomach tumor, colon tumor, rectal tumor, and thyroid tumor. Weaker signals were in adrenal tumor and normal adrenal, peratoid tumor, and lymphoma tumor. Weakest signals were observed in normal liver, normal esophagus, normal stomach, normal colon, normal rectum, normal thyroid, and normal lymphoma. The Stomach and Colon Tumor Blots showed signals consistent with those observed in the panel blots for stomach and colon tissue.

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#### Example 6

##### Chromosomal Assignment and Placement of Murine Zsig9

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Murine Zsig9 was mapped in mouse to chromosome 10 using the commercially available mouse T31 whole genome radiation hybrid (WGRH) panel (Research Genetics, Inc., Huntsville, AL) and the Map Manager QT linkage analysis program. At  $P = 0.0001$ , murine Zsig9 linked proximal to D10Mit136 at 62.0 cM with a LOD score of 4.3. This is a known region of synteny or linkage conservation with regions of human chromosome 12.

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The T31 WGRH panel contains PCRable DNAs from each of 100 radiation hybrid clones, plus two control DNAs (the 129aa donor and the A23 recipient). For the mapping of murine Zsig9 with the T31 WGRH panel, 20  $\mu$ l reactions were set up in PCRable 96-well microtiter plates



(Stratagene, La Jolla, CA) and used in "RoboCycler Gradient 96" thermal cyclers (Stratagene). Each of the 102 PCR reactions consisted of 2  $\mu$ l 10X KlenTaq PCR reaction buffer (CLONTECH Laboratories, Inc., Palo Alto, CA), 1.6  $\mu$ l dNTPs mix (2.5 mM each, PERKIN-ELMER, Foster City, CA), 1  $\mu$ l sense primer, (SEQ ID NO:22), 5' TCG CGC GAG AGT TTG GAG 3', 1  $\mu$ l antisense primer, (SEQ ID NO:23), 5' CCC AGC TTC CCG CAC TTA 3', 2  $\mu$ l "RediLoad" (Research Genetics, Inc., Huntsville, AL), 0.4  $\mu$ l 50X Advantage KlenTaq Polymerase Mix (Clontech Laboratories, Inc.), 25 ng of DNA from an individual hybrid clone or control and x  $\mu$ l ddH<sub>2</sub>O for a total volume of 20  $\mu$ l. The reactions were overlaid with an equal amount of mineral oil and sealed. The PCR cyclers conditions were as follows: an initial 1 cycle 5 minute denaturation at 94°C, 35 cycles of a 1 minute denaturation at 94°C, 1 minute annealing at 62°C and 1.5 minute extension at 72°C, followed by a final 1 cycle extension of 7 minutes at 72°C. The reactions were separated by electrophoresis on a 2% agarose gel (Life Technologies, Gaithersburg, MD). The use of the surrounding markers positions murine Zsig9 in the 51-62 centiMorgan (cM) region on mouse chromosome 10 map. Other genes of interest which lie in or near this region are glioma-associated oncogene homolog, myogenic factors 5 and 6, mast cell growth factor, insulin-like growth factor, and tumor rejection antigen-1.